PART I - ADMINISTRATIVE

Section 1. General administrative information

Title of project

Intracytoplasmic Sperm Injection: Genetic Retrieval From Single Sperm

BPA project number: 20043
Contract renewal date (mm/yyyy): 10/2000 ☒ **Multiple actions?**

Business name of agency, institution or organization requesting funding
University of Idaho

Business acronym (if appropriate) UI

Proposal contact person or principal investigator:

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NPPC Program Measure Number(s) which this project addresses
7.2, 7.4D, 7.4E (The 1994 Columbia River Basin Fish and Wildlife Program)

FWS/NMFS Biological Opinion Number(s) which this project addresses

Other planning document references

Short description
Develop methodology to retrieve the genetics from a single sperm by microinjection into an egg.

Target species
chinook salmon (Oncorhynchus tshawystcha)
steelhead (Oncorhynchus mykiss)

Section 2. Sorting and evaluation

Subbasin
Systemwide

Evaluation Process Sort

CBFWA caucus	Special evaluation process	ISRP project type
Mark one or more caucus	If your project fits either of these processes, mark one or both	Mark one or more categories
☒ Anadromous fish ☐ Resident fish ☐ Wildlife	☒ Multi-year (milestone-based evaluation) ☐ Watershed project evaluation	☐ Watershed councils/model watersheds ☐ Information dissemination ☐ Operation & maintenance ☐ New construction ☒ Research & monitoring ☐ Implementation & management ☐ Wildlife habitat acquisitions

Section 3. Relationships to other Bonneville projects

Umbrella / sub-proposal relationships. List umbrella project first.

Project #	Project title/description
	None

Other dependent or critically-related projects

Project #	Project title/description	Nature of relationship
9703800	Listed Salmonid Stocks Gamete Preservation	ICSI of salmonid sperm will interact with the development of a sperm bank by ultimately providing insurance against accidental thawing of the cryopreserved materials
	Enhancement of Samonid Gamete Quality by Manipulation of Intracellualar ATP	CO-PI; both projects have a goal of increasing the probability of retrieving the genetic information from sperm
	Endocrine Control of Ovarian Development in Salmonids	A participant of the UI/WSU Fish Reproduction Program
	Analyzing Genetic and Behavioral Changes During Samonid Domestication	A participant of the UI/WSU Fish Reproduction Program

	Induction of Precocious Sexual Maturity and Enhanced Egg Production in Fish	A participant of the UI/WSU Fish Reproduction Program
	Viral Vaccines and Effects on Reproductive Status	A participant of the UI/WSU Fish Reproduction Program

Section 4. Objectives, tasks and schedules

Past accomplishments

Year	Accomplishment	Met biological objectives?
	None	

Objectives and tasks

Obj 1,2,3	Objective	Task a,b,c	Task
1	Determine the pretreatment of sperm and the timing of the injection of the sperm relative to egg activation that maximizes male pronuclear development	a	Describe the temporal changes in the decondensation of the sperm chromatin and the development of the male pronucleus following normal fertilization
		b	Pretreat sperm with dithiothreitol and/or sonication before microinjection into the eggs. The measurable endpoint of this task is the proportion of eggs in which a male pronucleus is formed.
		c	Inject the sperm into eggs at varying times following activation. The measurable endpoint will be the proportion of eggs in which a male pronuclues is formed.
2	Produce viable offspring from the injection of a single sperm into an activated egg	a	Inject sperm with the gene for pigmentation into activated, albino trout eggs (the protocol for sperm pretreatment and the time of injection will be dictated by the results of objective 1). The measurable endpoint will be normal, pigmented fry.

		b	Inject steelhead sperm into activated steelhead eggs. The measurable endpoint will be normal, diploid fry.
		c	Inject chinook sperm into activated chinook eggs. The measurable endpoint will be normal, diploid fry.

Objective schedules and costs

Obj #	Start date mm/yyyy	End date mm/yyyy	Measureable biological objective(s)	Milestone	FY2000 Cost %
1	10/2000	9/2001	The proportion of eggs in which the male pronuclues has developed following the injection of a single sperm	Producing a protocol for the pretreatment of the sperm and the timing of injection following egg activation for the microinjection of single sperm into salmonid eggs	37.80%
2	10/2000	9/2001	The proportion of normal, functional offspring produced by the intracytoplasmic injection of salmonid sperm into activated eggs	The ability to produce offspring from a single sperm cell; the ability to retrieve the genetics from a single sperm cell; teach fisheries personel to microinject sperm into activated eggs.	62.20%
				Total	100.00%

Schedule constraints
None

Completion date
9/2001

Section 5. Budget

FY99 project budget (BPA obligated): $0

FY2000 budget by line item

Item	Note	% of total	FY2000
Personnel	PI (4.0 mo); Scientific Aide (12 mo); Graduate Student (9.0 mo); Undergrad (320 hrs)	%35	77,696
Fringe benefits	28.5% for PI and Scientific Aide; 1.0% for students	%8	16,940
Supplies, materials, non-expendable property		%11	24,000
Operations & maintenance	equipment repair	%2	4,240
Capital acquisitions or improvements (e.g. land, buildings, major equip.)		%4	8,765
NEPA costs		%0	0
Construction-related support		%0	0
PIT tags	# of tags:	%0	0
Travel		%1	3,000
Indirect costs		%31	69,124
Subcontractor		%0	0
Other	Administrative and Aquaculture Core Facilities	%9	20,000
TOTAL BPA FY2000 BUDGET REQUEST			$223,765

Cost sharing

Organization	Item or service provided	% total project cost (incl. BPA)	Amount ($)
None		%0	
		%0	
		%0	
		%0	
Total project cost (including BPA portion)			$223,765

Outyear costs

	FY2001	FY02	FY03	FY04
Total budget	$234,953	$246,701	$259,036	$271,988

Section 6. References

Watershed?	Reference
☐	Flahrety, S.P., D. Payne, N.J. Swann and C.D. Matthews. 1995. Assessmnt of fertilization failure and abnormal fertilization failure after intracytoplasmic sperm injection (ICSI). Reprod Fertil Dev 7:197-210.
☐	Gausen, D. 1993. The Norwegian gene bank programme for Atlantic Salmon. In: Cloud, J.G. and Throgaard, G.H. (eds) Genetic conservation of salmonid fishes. Plenum Press, New York pp181-188.
☐	Kimura, Y. and R. Yanagimachi. 1995. Intracytoplasmic sperm injection in the mouse. Biol Reprod 52:709-720.
☐	Palermo, G.D., H. Joris, P. Devroey and A.C. Van Strirteghem. 1992. Pregnancies after intracytoplasmic injection of a single spermatozoon into an oocyte. Lancet. 340:17-18.
☐	Tesarik, J. an M. Sousa. 1995. More than 90% fertilization rates after intracytoplasmic sperm injection and artifical induction of oocyte activation with calcium ionophore. Fertil Steril 63:343-349.
☐	Uehara, T. and R. Yanagimachi. 1976. Microsurigical injection of spermatozoa into hamster eggs with subsequent transformation of sperm nuclei into male pronuclei. Biol Reprod 15:467-470.
☐	Van Steirteghem, A.C., Z. Nagy, H. Joris, J. Liu, C. Straesen, J. Smitz, A. Wisamto and P. Devroey. 1993. High fertilization and implantation rates after intracytoplasmic sperm injection. Hum. Reprod. 8:1061-1066.
☐	Yanagida, K., R. Yanagimachi, S.D. Perreault and R.G. Kleinfeld. 1991. Thermostability of sperm nuclei assissed by microinjection into hamster oocytes. Biol Reprod. 44:440-447.
☐	

PART II - NARRATIVE

Section 7. Abstract

The genetic contributions of male fish from threatened and endangered species are lost if their sperm cells are unable to fertilize eggs. A similar biological problem, human male factor infertility, exists in medicine. The human reproductive problem has been solved by the development of intracytoplasmic sperm injection (ICSI); normal human offspring are now routinely produced by the injection of a single sperm cell into an egg. Since the post-fertilization cellular events, sperm nuclear decondensation and the formation of the male pronucleus, appear to be very similar between mammals and fish, the overall objective of this proposed investigation is to produce fish by injecting a single sperm into an egg. **The hypothesis to be tested is that the mechanical introduction of a sperm into the cytoplasm of an activated egg will result in the decondensation of the chromatin within the sperm nucleus, in the development of a morphologically normal male pronucleus, and in the production of a fully functional zygote.** The experiments

proposed for FY2000 are designed to develop the methodology of sperm intracytoplasmic injection for fish and to demonstrate that the DNA of the injected nucleus contributes to the genome of the resultant zygote. Ultimately, the ability to produce fish by ICSI is expected to provide the means to retrieve the genetics of sperm 1) from infertile or subfertile males, 2) that has been prematurely activated during collection, 3) that was cryopreserved and accidentally thawed, or 4) from dead or dying males.

Section 8. Project description

a. Technical and/or scientific background

In the normal process of fertilization, the genetic information of sperm is transferred to the egg with the formation of a new, genetically unique individual. As a result, the sperm contributes a chromosomal set to the resultant embryo and the genetics represented by the sperm is passed to the next generation. This normal method of genetic retrieval from a sperm cell in fish is dependent on the ability of the sperm to move into the micropyle of the chorion and fuse with the plasma membrane of the egg. Since factors such as sperm concentration of the milt, level of adenosine triphosphate (ATP) in the sperm, the percent motility of the sperm upon activation, or the structural integrity and morphology of the sperm dictate or define the ability of the sperm to successfully fertilize a egg, the genetic contribution of a male to the gene pool of future generations can be compromised because of the inability of its sperm to fertilize eggs.

The cryopreservation of sperm is being used by a number of different countries for the development of gene banks to preserve the genetic diversity of fish populations (for example see Gausen, 1993). This procedure is attractive because the sperm cells are relatively easy to collect, and sperm are readily cryopreserved. Although the cells are stressed during the freezing and thawing periods and there is a reduction in the fertility of frozen sperm as compared to fresh sperm, this technology provides the means to store unique genes of fish stocks for decades and centuries, and the genetics of the sperm can be retrieved easily by fertilizing eggs with the thawed sperm. As previously discussed, the reintroduction of this stored genetics is dependent upon the successful fertilization of oocytes with the thawed sperm. Even though only one sperm cell enters each oocyte or egg, approximately 200,000 motile sperm are normally required per egg for maximum fertility (personal observation). Since the freezing and thawing of sperm associated with sperm banking result in cellular injury and a decline in fertility, fresh milt samples containing sperm with little or no motility upon activation are usually culled prior to cryopreservation and are not included in the sperm bank.

The collection of sperm from wild male salmonids is not always done under ideal conditions. As a result, water can inadvertently contaminate the milt as it is being collected and induce the activation of the sperm. Additionally, unlike a hatchery situation, sperm collected from wild males may not always be collected at the time of maximal fertility. In both of these cases, the quality of the milt, the percent motility of the

sperm or the quality of the sperm may not be sufficient to warrant its cryopreservation and storage. As noted above, sperm (and the genetics of the sperm) may be culled from the sperm bank because the probability of retrieving the genetics of the sperm by fertilization post-thaw is nil.

A number of dead or dying males are present in the spawning regions throughout the course of the reproductive season. These males represent the normal outcome for these individuals following sexual reproduction. Although sperm is generally present in these males, this sperm is not collected for long-term storage because under normal conditions of fertilization, these sperm are not motile and are infertile.

Once the sperm are frozen and are a part of the sperm bank, the long term storage of these cells and the genetics that they represent requires that the samples be continually maintained in liquid nitrogen at -196^{o} C. At present, the accidental loss of liquid nitrogen and the thawing of the stored materials followed by refreezing will result in a loss of fertility of the stored sperm. Additionally, even though a single straw of frozen milt may contain billions of sperm and the genetics of a male may be represented by very few straws of frozen milt, thawing a straw negates the usefulness of the sperm cells not used in the fertilization process. The ability to retrieve the genetics from sperm that have been frozen, thawed, and refrozen would provide insurance for the gene bank and increase the efficient use of the stored materials.

In human reproductive medicine, male factor infertility is generally due to a low number of sperm or a low number of motile sperm in the ejaculate. This reproductive problem has been successfully treated using intracytoplasmic sperm injection (ICSI; Palermo, 1992; Van Steirteghem et al., 1993; Flaherty et al., 1995); a zygote is produced by injecting a single sperm directly into the egg. This technology provides the means by which sperm from males with a low sperm count can be used to fertilize eggs. In fact the nucleus derived for this procedure can be obtained from either ejaculated or non-ejaculated sperm or from spermatids. At present, this procedure is used routinely in human reproductive medicine to combat male infertility, and there is no evidence to indicate that this procedure increases the risk of abnormal development or congenital abnormalities (Flaherty et al., 1995). In fact many fertility clinics use ICSI instead of in vitro fertilization because of the high rate of success (Tesarik and Sousa, 1995).

Although ICSI was developed over twenty years ago (Uehara and Yanagimachi, 1976) and it has been demonstrated to be highly successful in mammals (see Kimura and Yanagimachi, 1995, for the mouse and Tesarik and Sousa, 1995, for the human as examples) ICSI does not appear to have been used previously in fish. However, the successful development of male pronuclei in 100% of the hamster eggs microinjected with talipia sperm (Yanagida et al., 1991) suggests that this technology can be transferred from mammalian reproductive biology to fisheries to provide the means of retrieving the genetics from single sperm. Additionally, the similarity of the cellular processes associated with successful fertilization, the decondensation of the sperm head and the subsequent formation of the male pronucleus, between mammals and fish, support the argument that ICSI should be a viable alternative to fertilization in fish.

b. Rationale and significance to Regional Programs

A zygote is the product of a single sperm nucleus entering an egg. Normally, successful fertilization is dependent upon motility and sperm density or concentration. The development of ICSI for salmonid eggs would negate these normal requirements for fertilization and provide the means of retrieving the genetics from subfertile or infertile sperm.

Rationale

ICSI is currently being utilized in fertility clinics throughout the world to produce human offspring. This procedure has a high success rate and is being used in place of *in vitro* fertilization for assisted human reproduction. Each resulting offspring requires only a single sperm, and there is no evidence that ICSI increases congenital abnormalities. Since the cellular mechanisms associated with the fertilization process (sperm nuclear decondensation and formation of the male pronucleus) are similar in mammals and fish, it is highly probable that normal, functional salmonids can be produced by ICSI.

Significance

The overall goal of this proposed investigation is expected to provide a positive impact on the genetic conservation of fishes within the region that are threatened or endangered. The development of ICSI for fish is expected to provide the methodology by which the genetics of sperm that are presently infertile can be incorporated into future generations.

Using ICSI, it is expected that the genetics of sperm derived from the following conditions can be utilized in the production of offspring.

1. **sperm obtained from subfertile to infertile males (males with dilute milt or low sperm count)**
2. **sperm that that has been previously activated or is non-motile**
3. **sperm that was not properly stored (for example, held at –80^{o} C for an extended period of time)**
4. **sperm that has been frozen, thawed, and refrozen**
5. **sperm from dead or dying males**

The successful outcome of this project will provide us with a method to retrieve the genetics from males that have very few motile sperm, to use sperm that has been activated during the collection process, or to use sperm from cryopreserved samples that have been thawed and refrozen. This technology will be transferred from existing methodology that has been developed to provide a solution to human males that are infertile because of a low sperm count. Although this type of technology is not practical for the normal production of fish, in those cases in which the genetics of a male with poor sperm quality needs to be maintained or in the case in which the samples maintained in a sperm bank have been compromised by accidental thawing, then the additional time and effort required by this proposed approach is justified as a means of genetic retrieval.

Additionally for species like the white sturgeon for which protocols for successful cryopreservation of spermatozoa are not currently available, ICSI could potentially provide the means of retrieving the genetics from sperm that has been cryopreserved. As a result, sperm from white sturgeon could be cryopreserved even though the thawed sperm was infertile if the genetics could be retrieved by ICSI. This time advantage (collecting and storing sperm in liquid nitrogen before a protocol for sturgeon sperm cryopreservation is developed) may provide a means of saving genetic information for this species that may otherwise be lost.

c. Relationships to other projects

Listed Salmonid Stocks Gamete Preservation, a project from the Nez Perce Tribe, has proposed to develop a germ plasm repository or sperm bank for chinook salmon. The development of the methodology proposed in this project - to use a single sperm to fertilize an egg regardless of its motility - will increase the genetic resources available for banking (any sperm can be included if motility is not a criterion for banking) and ICSI of salmonid sperm will provide insurance against accidental thawing of the cryopreserved materials (if successful, we should be able to use sperm that has been accidentially thawed).

Another proposed project from the University of Idaho, Enhancement of Samonid Gamete Quality by Manipulation of Intracellualar ATP, is also designed to support efforts in the development of a salmonid gene bank.

In addition to the project listed above, the following are proposed projects of the UI/WSU Fish Reproduction Program:
Endocrine Control of Ovarian Development in Salmonids
Analyzing Genetic and Behavioral Changes During Samonid Domestication
Induction of Precocious Sexual Maturity and Enhanced Egg Production in Fish
Viral Vaccines and Effects on Reproductive Status

All members of the UI/WSU Fish Reproduction Program will interact by participating in a joint seminar program and by sharing core facilities.

d. Project history (for ongoing projects)

None (new project)

e. Proposal objectives

The overall objective of this proposed investigation is to produce viable offspring by injecting single sperm cells into activated, salmonid oocytes.

The specific objectives of this FY2000 project are as follows:

1. **To identify which treatments of sperm prior to injection into activated eggs maximize sperm nuclear decondensation and formation of the male pronucleus.**
2. **To produce viable young from the microinjection of single sperm nuclei into individual oocytes**

The protocols developed in year 1 will be utilized to examine the types of conditions in which ICSI can be beneficial. For example, this methodology will be examined to determine if it can be used to produce offspring from sperm that have been previously activated (non-motile) and from sperm that have been damaged or derived from dead or dying males.

The completion of objections for year 1 will lead to the following objectives in subsequent years.

Year 2. To produce viable offspring from sperm nuclei derived from previously activated, non-motile sperm and from sperm that have been frozen and thawed multiple times.

Year 3. To determine the fertility of sperm from dead or dying salmon.

Year 4. To produce viable young from the transfer of single, sturgeon nuclei derived from frozen sperm into individual oocytes

Year 5. To produce offspring from sperm stored for varying lengths of time at -80^0 C, and from sperm that has been freeze dried.

f. Methods

Experimental Design

Experiment 1. The objective of this experiment is to identify which pretreatment conditions used for mammalian ICSI and the timing of injection will maximize nuclear decondensation and male pronuclear in salmonids.

The hypothesis to be tested is that the chromatin of isolated sperm nuclei injected into activated fish eggs will undergo decondensation and become organized into the male pronucleus. Since nuclear decondensation is dependent upon the reduction of disulfide bonds and since sonication is expected to remove the tail and permeablize the sperm membrane, pretreatment of the sperm with dithiothreitol and sonication are hypothesized to increase the incidence on male pronuclear formation following ICSI.

The factors that will be examined in experiment 1 are (a) the pretreatment of the sperm (pretreatment of the sperm with dithiothreitol [DTT; 5 mM] and/or sonication [one

minute burst at 110 W power output with an Ultrasonic sonicator; Bronwill Model BP-II, Bronwill Scientific, Rochester, NY]), and (b) the time interval between egg activation and sperm injection. This experiment is designed as a 4 x 5 factorial. The treatment groups are no DTT / no sonication, DTT, sonication, and DTT + sonication with injection of the sperm immediately following, 10, 20, 30 or 40 minutes post-activation (oocyte activation will be induced by fertilizing the eggs with uv-irradiated sperm). The positive control group will be eggs fertilized with viable sperm with and without the injection of a sperm (the resultant embryos are expected to be diploid and triploid). The negative control group will be eggs fertilized with uv-irradiated sperm with no injected sperm (the resultant embryos are expected to be haploid). After pretreatment, the sperm will be washed 3X by centrifugation (1000 x g, 10 min) in a modified Cortland solution. The sperm to be injected will be prepared by placing 1 μL of sperm solution (10^9 sperm per mL) in 10 μL of 10% polyvinylpyrrolidone (PVP) in Ham's F-10 medium. A single sperm nucleus will be drawn up into a glass micropipet (inside diameter 2.5 μm). The eggs to be injected will be activated by fertilizing them with uv-irradiated sperm. Following activation at varying time intervals, the micropipette will be inserted through the micropyle, and the blastodisc will be impaled and the sperm nucleus injected. The injected eggs will be incubated individually at 11^o C until the time of fixation.

The time at which the blastodisc will be fixed and observed will be determined from a preliminary experiment (see below). At the time of assay, the blastodisc will be dissected free of the yolk sac, compressed between a coverslip and slide, fixed in methanol: acetic acid (3:1) and stained with 1% aceto-lacmoid. The fixed bastodisc of the fertilized eggs will be observed using phase contrast microscopy. From the histological analysis of the resultant preparation, the degree of pronuclear development will be determined as described and illustrated by Yanagida et al. (1991).

The endpoint for this experiment will be the morphology of the injected sperm nucleus and its changes as compared to the positive control (normal fertilization) group. The expectation is that the results of this experiment will provide the best set of pretreatment conditions for salmonid sperm for the ICSI process.

Possible pitfalls:
My laboratory has a long history of producing haploid embryos by fertilizing eggs with uv-irradiated sperm and microinjecting fish zygotes (to deliver DNA). Therefore, the proposed experiment should not be compromised by the lack of technical experience. The mechanics of this experiment will be worked out first using rainbow trout prior to the spawning seasons for steelhead and chinook.

The most serve potential pitfall is that individual sperm nuclei microinjected into fish eggs do not develop into a pronucleus and that none of the pretreatments have any positive effect. If this outcome occurs, the experiment will be repeated and the sperm will be pretreated by crushing, repeated freezing and thawing, use of anionic detergents, proteases and other disulfide reducing agents. Since 100% of the tilapia sperm nuclei treated with sonication only developed into a pronucleus when injected into hamster

oocytes (Yanagida et al., 1991), it seems very likely that our initial treatments will result in pronuclear development.

A second potential pitfall is that our proposed method of following pronuclear development (based on the methodology of the mammalian literature) may not be appropriate for fish. Our alternative will be to follow the cellular events using electron microscopy.

In order to be informed about the normal events and timing of pronuclear development in salmonids, fertilized eggs at regular time intervals post-fertilization will be collected for both chinook salmon and steelhead prior to the initiation of this proposed investigation. At the time of collection, the chorion will be removed by dissection (in a calcium-free fish Ringers solution using sharpened #5 forceps) and the blastodisc will be trimmed from the yolk sac and fixed as described above. This preliminary study will provide us with the normal timing of pronuclear development and it will allow us to test our methodology before the initiation of the experiment. This preliminary study and the histological analysis of the experiment will be conducted by the graduate student.

Experiment 2. The objective of this experiment is to produce viable offspring from the injection of a single sperm cell into an activated, salmonid egg.

The hypothesis to be tested is that the injected sperm nucleus will contribute to the development of the resultant embryo.

Experiment 2a.
To test this hypothesis, in experiment 2a, sperm from male rainbow trout that are homozygous for normal pigmentation will be injected into activated eggs (method of sperm preparation and injection was described in experiment 1) derived from albino females (eggs from albino females activated by fertilizing with uv-irradiated sperm from albino males). The resultant embryos will be incubated individually at 11^{o} C. At 140 days of age, the resultant embryos will be examined to determine their pigmentation and the presence of developmental abnormalities.

The expected results of this experiment are that the offspring will have normal pigmentation derived from the injected sperm DNA and that there will be few or no congenital abnormalities. The use of rainbow trout in this part of the experiment is justified by the need for a simple genetic, color marker and for the need to develop the expertise in sperm delivery (sperm injection) before the spawning season for steelhead and chinook.

Experiment 2b.
In experiment 2b, single sperm nuclei from steelhead and chinook salmon will be injected into activated eggs of the same species. The method of sperm preparation and injection will be the same as described in experiment 1 (the methodology that proved successful in experiment 1). The treatment groups in this experiment are i) eggs fertilized with uv-

irradiated sperm, ii) eggs fertilized with normal sperm, and iii) eggs fertilized with uv-irradiated sperm + injection with a single sperm. The resultant embryos will be incubated at 11^{o}C. At 200 days of age (in FY2001), the ploidy of five surviving fish from each group will be determined by flow cytometry of a blood sample

It is expected that activated eggs not injected with a sperm nucleus or activated eggs injected with a sperm nucleus that do not develop a functional male pronucleus will be haploid and will not develop through hatching. Alternatively, activated eggs injected with a sperm nucleus and eggs fertilized with a normal sperm are expected to be diploid and to develop normally. The resultant offspring from this study will be reared to sexual maturity and subsequently spawned (to determine if they are reproductively functional).

Possible pitfalls:
There are no perceived pitfalls for experiment 2. This experiment will be conducted by the Scientific Aide and the PI.

g. Facilities and equipment

Research facilities available for use campus-wide include scanning and transmission electron microscopes. The following multi-user equipment available for our use in the department includes: Amsco steam autoclave, MilliQ cartridge purification system for tissue culture grade water, photographic supplies and darkroom, Zeiss compound microscope with epifluorescence, Packard beta scintillation counter, Beckman L7 and L8M ultracentrifuges, Savant Speedvac Concentrator, Bio-tek ELISA plate reader, Brandell cell harvestor and a thermal cycler. In addition, our department (Biological Sciences) has a Core Molecular Laboratory.

The laboratory space is located in Gibb Hall (rooms 236 and 241). Room 241 is sectioned into two areas to isolate specific functions. The main part is 20' X 25' and is used for general laboratory purposes; the other part is 10' X 9' and is used as a sterile room for activities pertaining to cell and tissue culture. The following equipment is located in the laboratory and is available for use during this investigation; Beckman GPR centrifuge, EAC laminar flow hood, chemical hood, Corning 140 pH meter, Westcor vapor pressure osmometer, Sartorius Analytical top-loading balance, Nikon Diaphot inverted microscope, Wild and Olympus stereomicroscopes, Leitz dual micromanipulator, DKI vertical pipette puller, microforge, Olympus BH-2 light microscope and camera, Beckman Microfuge, refrigerator/freezer, Gilson constant temperature water bath, Rainin and Eppendorf digital pipettors, glassware drying oven and Hoefer SDS-PAGE and agarose gel units. Room 236 is a wet lab; it is adjacent to the main lab and has three living stream units and two eight-tray Heath incubation units.

h. Budget

Salaries
The salaries are to support a scientific aid (full time), a graduate student (academic year/9 months) and the PI (part-time/4 months). The graduate student will describe the normal developmental events associated with the formation of the male pronucleus in salmonids; this individual will take the lead in the electron microscopy if this is required to follow pronuclear development. The scientific aid will be experienced in cell microinjection and will develop the proposed protocols for ICSI in salmonids. The PI will be responsible for all phases of the investigation and the writing of the resultant publications. Salary is also requested for part-time help of an undergraduate; this individual will clean glassware and monitor the embryos during the incubation phase.

Materials and Supplies
These requested funds will be utilized to purchase supplies (1) for the ultra-structural analysis of fertilization (fixatives, imbedding materials, slides and stains, electron microscope time), (2) treatment of the sperm, and (3) for the microinjection of the sperm (media and special glass to make the micropipettes)

Operations and Maintenance
The funds that are requested represents an estimate of the repair costs for the equipment that will be used to support this proposed investigation ($4240)

Equipment
The cost of a pico-injector ($3,800) is requested. My laboratory has all equipment/instrument needs for intracytoplasmic injection; the addition of a pico-injector, an instrument that can automatically control the injection of pL quantities of fluid with a glass micropipette, would increase our accuracy and our consistency in the microinjection process.
A new base and lighting system ($4,965) for my Wild stereo microscope is requested in order to upgrade the equipment to be used for the injection process.

Travel
Travel is requested for two people to attend a scientific meeting (estimated at $1500 per person)

Other
These funds are budgeted to support a core fish rearing facility ($15,000) and a core administrative unit ($4,000). The Administrative Core laboratory will integrate the activities of the Fish Reproduction Program and the Center for Reproductive Biology for administration of grants, core laboratories, and activities such as seminars, workshops, and retreats. The integration of the different projects and research activities requires the Administrative Core laboratory support requested. The Aquaculture Core laboratory provides the faculty with services required for the maintenance of fish populations for the proposed research. This is a critical aspect of the proposed studies.

Section 9. Key personnel

1. Present Position:
Professor of Zoology
Department of Biological Sciences
University of Idaho
Moscow, Idaho 83844-3051

3. Education:
Ph.D. (Reproductive Physiology/Endocrinology) University of Wisconsin Madison (1974)
MS (Reproductive Physiology/Endocrinology) University of Wisconsin-Madison (1968)
BS (Physiology/Pre-Veterinary Medicine) West Virginia University (1966)

3. Employment history:
i) Professor/Associate Professor/Assistant Professor of Zoology: University of Idaho (August 1977 - present)
ii) Visiting Professor: University of Minnesota (July 1983 - July 1984)
iii) Assistant Professor: Johns Hopkins University (October 1976 - August 1977)
iv) Postdoctoral Fellow: Johns Hopkins University (October 1974 - October 1976)
v) Research Assistant: University of Wisconsin-Madison (September 1971 - October 1974)
vi) Officer: U.S. Navy (November 1968 - September 1971)
vii) Research Assistant: University of Wisconsin-Madison (July 1966 - November 1968)

4. Selected publications:

Bencic, D. C., M. Krisfalusi, J.G. Cloud and R.L. Ingermann. 1998. ATP levels of chinook salmon (*Oncorhynchus tshawytscha*) sperm following in vitro exposure to various oxygen tensions. *Fish Physiology and Biochemistry* (In Press)

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Thorgaard, G.H. and J.G. Cloud. 1993. Reconstitution of genetic strains of salmonids using biotechnical approaches. In:"Genetic Conservation of Salmonid Fishes". (J.G. Cloud and G.H. Thorgaard, eds.) Plenum Press, New York, pp. 189-196.

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Section 10. Information/technology transfer

Information gained in this proposed investigation will be published in a peer-reviewed journal. Additionally, the methodology of ICSI for salmonids will be added to my annual summer workshop on the cryopreservation of salmonid sperm.

Congratulations!